International Journal of Odonatology, 2014
Vol. 17, Nos. 2–3, 135–147, http://dx.doi.org/10.1080/13887890.2014.950698

Taylor & Francis
Taylor & Francis Group

New EPIC nuclear DNA sequence markers to improve the resolution of phylogeographic studies of coenagrionids and other odonates

Sónia Ferreira[a,b,c*], M. Olalla Lorenzo-Carballa[c,d], Yusdiel Torres-Cambas[e], Adolfo Cordero-Rivera[d], David J. Thompson[c] and Phillip C. Watts[c,f]

[a]*CIBIO – Centro de Investigação em Biodiversidade e Recursos Genéticos, InBIO Laboratório Associado, Universidade do Porto, Vairão, 4485-661 Vairão, Portugal;* [b]*Departamento de Biologia da Faculdade de Ciências da Universidade do Porto, Rua Campo Alegre, 4169-007 Porto, Portugal;* [c]*Institute of Integrative Biology, Biosciences Building, Crown Street, University of Liverpool, Liverpool, L69 7ZB, UK;* [d]*Grupo de Ecoloxía Evolutiva e da Conservación, Departamento de Ecoloxía e Bioloxía Animal, Universidade de Vigo, EUE Forestal, Campus Universitario, 36005, Pontevedra, Spain;* [e]*Facultad de Ciencias Naturales, Universidad de Oriente, Patricio Lumumba s/n, Santiago de Cuba, CP 90500, Cuba;* [f]*Department of Biology, University of Oulu, PO Box 3000, FI–90014 Oulu, Finland*

(*Received 2 May 2014; accepted 25 July 2014*)

While phylogeographic data provide valuable information to inform conservation plans, there are comparatively few Odonata phylogeographic studies. This lack of research is partially due to a lack of independent DNA markers with appropriate levels of polymorphism that PCR-amplify in a range of species. We followed an exon-primed, intron-crossing (EPIC) PCR strategy to develop five new, polymorphic nuclear DNA sequence loci (six distinct DNA fragments) for the southern damselfly *Coenagrion mercuriale*. These markers were: cell division cycle 5 protein (CDC5), arginine methyltransferase (PRMT), acetylglucosaminyl-transferase (AgT), myosin light chain (MLC) and phosphoglucose isomerase (PGI). Between three and five of these new markers could be PCR-amplified in five other species from the genus *Coenagrion*; one locus (PRMT) can be used in 26 other species of odonates that we examined, including three species of Anisoptera belonging to the genus *Onychogomphus*. These new nuclear genetic markers will be useful for phylogeographic studies in a range of odonate species, but also for phylogenetic studies, providing a particularly useful complement to the existing mitochondrial and nuclear loci.

Keywords: Coenagrionidae; *Coenagrion mercuriale*; dragonfly; genetic diversity; introns; multilocus approach; non-model organism; nuclear DNA loci; Odonata; phylogeny

Introduction

Phylogeographic studies yield insights into the suite of historic processes that have shaped the contemporary distribution of genetic diversity (Avise, 2000). Such information assists effective conservation management by providing fundamental data about evolutionarily significant

*Corresponding author. Email: Hiporame@gmail.com

units (ESUs), thereby allowing prioritization of areas/populations that represent high value for conservation (Moritz, 2002).

Regions of mitochondrial DNA (mtDNA) are still the most widely used molecular-genetic markers to assess phylogeographic structure in animal populations (Avise, 2000, 2009; Ballard & Whitlock, 2004). The popularity of mtDNA for phylogeographic studies is largely due to (1) its higher rate of mutation than in most nuclear genes; (2) a predominantly maternal inheritance that generally excludes recombination as a source of diversity and ensures homoplasmy; and (3) a conserved and tightly packed gene content that allows development of universal primers for PCR (e.g. Simon et al., 1994). While the mutation rate varies among mitochondrial genes, resulting in distinct phylogenetic resolution power, the use of mitochondrial genetic markers can be problematic to interpret. For example, the phylogeographic history of an organelle (such as the mitochondrion) may differ from an organism's history due to selective sweeps and introgression, or due to demographic attributes, such as population size, dispersal ability and/or sex-biased dispersal (Ballard & Whitlock, 2004; Galtier, Nabholz, Glémin, & Hurst, 2009; Hare, 2001). Amplification of numts (nuclear mitochondrial DNA; Lopez, Yuhki, Masuda, Modi, & O'Brien, 1994), where whole or fragments of mitochondrial genes are incorporated into the nuclear genome, might constitute an additional problem by providing a heteroplasmic signal (in the absence of true heteroplasmy). Nevertheless, with careful analysis numts can be used to uncover complex evolutionary histories in the context of phylogeographic analysis (e.g. Miraldo, Hewitt, Dear, Paulo, & Emerson, 2012). Further problems with the use of mtDNA as a phylogeographic marker may arise in studies of arthropods due to indirect selection on mtDNA arising from linkage disequilibrium with maternally inherited symbionts (Hurst & Jiggins, 2005). Robust phylogenetic inference thus requires data from several independent genetic markers. In animals, such independent genetic loci must be derived from the nuclear genome as mitochondrial genes are physically linked and thus represent a single locus, except for some rare exceptions (e.g. Jiang, Barker, & Shao, 2013). Nonetheless, an apparently low rate of mutation found in most nuclear genes remains a major drawback for their use in phylogeography.

Introns (non-coding regions of genes located between exons) exhibit higher levels of polymorphism than exons (segments of genes that code for a peptide) and thus provide a potentially valuable source of nuclear genetic variation for phylogeographic studies. However, it is challenging to design reliable PCR primers in polymorphic regions of the genome, particularly if the goal is to amplify the locus in several species. The use of exon-primed intron-crossing (EPIC) markers resolves this issue by targeting conserved regions of the genome (i.e. the exons) to anchor primers that are used to PCR-amplify and sequence the adjacent introns (Lessa, 1992; Slade, Moritz, Heideman, & Hale, 1993). Indeed, EPIC markers have been successfully used in phylogeographic studies of a variety of taxa (e.g. Lohse, Sharanowski, & Stone, 2010; Aurelle et al., 2011; Jennings, Etter, & Ficarra, 2013).

Phylogeographic analyses of odonates remain relatively scarce compared with other animals (but see Artiss, 2004; Bernard, Heiser, Hochkirch, & Schmitt, 2011; Büsse et al., 2012; Jordan, Simon, Foote, & Englund, 2005; Kiyoshi, 2008; Kiyoshi & Sota, 2006). Notably, these odonate studies continue to rely upon a limited set of genes: cytochrome c oxidase I (COI), cytochrome *c* oxidase II (COII), NADH dehydrogenase 1 (ND1) and 16S ribosomal DNA (16S), and the nuclear ribosomal RNA gene region encompassing 18S, ITS1, 5.8S and ITS2. The present reliance on using mtDNA markers seems likely to continue with the recent publication of some entire odonate mitochondrial genomes (e.g. Chen et al., 2014; Lee et al., 2009; Lin et al., 2010; Lorenzo-Carballa, Thompson, Cordero-Rivera, & Watts, 2013; Wang et al., 2014). While these data offer the prospect of identifying universal PCR primers for the amplification of additional mtDNA genes in odonates, they do not resolve the key issue that all genes located on the mtDNA represent a single locus.

Just seven nuclear DNA sequence markers (18S, ITS1, 5.8S, ITS2, 28S, H3 and EF-1α) have been widely employed to quantify inter- and intra-specific relationships in odonates (Ballare & Ware, 2011). However, five of these markers (18S, ITS1, 5.8S, ITS2 and 28S) are physically linked to represent a single locus; moreover, the ribosomal coding sequences (5.8S, 18S and 28S) and histone 3 (H3) are conserved and thus largely uninformative for intraspecific phylogeographic studies (e.g. Cruickshank, 2002; Simon, Schierwater, & Hadrys, 2010). Elongation factor-1α (EF-1α) has been used to resolve odonate phylogenies (e.g. Carle, Kjer, & May, 2008; Jordan, Simon, & Polhemus, 2003) but its suitability to recover phylogeographic patterns is unclear (Simon et al., 2010). Essentially this leaves the ribosomal internal transcribed spacers (ITS1 and ITS2) that present intraspecific polymorphism in odonates (e.g. Dumont, Vanfleteren, De Jonckheere, & Weekers, 2005; Weekers, De Jonckheere, & Dumont 2001), thus there is a need to develop additional loci (1) to achieve statistical confidence and (2) because the ITS is a multicopy gene that can exhibit substantial intragenomic variability which obscures phylogeographic patterns (Harris & Crandall, 2000).

At present, only Lee and Lin (Molecular Ecology Resources Primer Development Consortium et al., 2012) have attempted to develop new nuclear DNA sequence markers in odonates. Eight EPIC loci were developed for *Euphaea* spp. (Euphaeidae) but their utility in other odonates was not tested. Thus, Odonata phylogeographic studies remain hampered by the lack of a set of independent, polymorphic nuclear DNA markers that are useful across a range of taxa. To redress this deficiency and to improve our understanding of odonate phylogeographic patterns using a multilocus approach, we developed five new nuclear DNA sequence loci in the southern damselfly *Coenagrion mercuriale*. These loci can be used in other zygopterans and in some anisopterans (*Onychogomphus* spp.), representing a wide taxonomic diversity encompassing regions in Europe, North Africa and Central America.

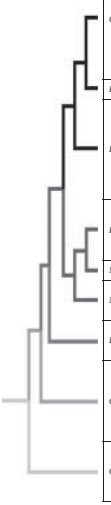
Material and methods

Target species and cross-amplification

Coenagrion mercuriale is one of the 19 Western Palaeartic species of the genus *Coenagrion*, and belongs to Coenagrionidae, the largest family of zygopterans with almost 1100 species distributed on all continents except Antarctica (Dijkstra & Kalkman, 2012). *Coenagrion mercuriale*'s distribution extends from the UK and Germany through France, Iberia, Italy and into North Africa (Dijkstra & Lewington, 2006). This species is classified in the IUCN red lists as Near Threatened in Europe (Kalkman et al., 2010) and as Endangered in North Africa (Samraoui et al., 2010). We sequenced 10 individuals of *C. mercuriale* (five from Europe and five from North Africa) to evaluate polymorphism in the newly developed markers.

Exploratory cross-amplification tests using the PCR primers for the new loci developed for *C. mercuriale* were performed in 26 other species of zygopterans and anisopterans (Table 1). Within the coenagrionids, we selected five species of *Coenagrion* (*C. caerulescens*, *C. ornatum*, *C. puella*, *C. pulchellum* and *C. scitulum*), one species of *Pseudagrion* (*P. sublacteum*) and six species of *Ischnura* (*I. hastata*, *I. ramburi*, *I. cervula*, *I. denticollis*, *I. kellicotii* and *I. posita*). Representatives of three other zygopteran families were *Protoneura capillaris*, *P. viridis*, *P. sanguinipes*, *Neoneura amelia*, *N. maria* and *Microneura caligata* (Protoneuridae; recently suggested to be part of Coenagrionidae *s. lat.*; Carle et al., 2008; Dijkstra et al., 2014), *Hypolestes clara* and *H. trinitatis* (Hypolestidae) and *Calopteryx haemorrhoidalis*, *C. xanthostoma*, *C. virgo* and *C. splendens* (Calopterygidae). Cross-amplification tests were also performed in three species of anisoptera from the genus *Onychogomphus* (*O. costae*, *O. forcipatus* and *O. uncatus*).

Table 1. Amplification success of six new EPIC nuclear loci in 27 odonate species displayed against a schematic representation of their putative evolutionary relationships (derived from Dijkstra et al., 2014). Data indicate the number of specimens sequenced, the primer pairs to PCR-amplify gene fragments and the Genbank accession numbers.



		CDC5			MLC			PRMT			AgT			PGI-8			PGI-1219		
Genus	**Species**	**N**	**Primers**	**Genbank**	**N**	**Primers**	**Genbank**	**N**	**Primers**	**Genbank**	**N**	**Primers**	**Genbank**	**N**	**Primers**	**Genbank**	**N**	**Primers**	**Genbank**
Coenagrion	*mercuriale*	10	F2-R2	KM248413-KM248432	10	F1u-R1cm	KM248443-KM248462	10	F4-R3	KM248503-KM248522	10	F2-R4	KM248393-KM248412	10	8F-382R	KM248463-KM248482	10	1219F-1455R	KM248483-KM248502
	ornatum				1	F1u-R1cm	KM276626	1	F4-R3	KM276639	1	F2-R4	KM276647	1	8F-382R	KM276651	1	1219F-1455R	KM276638
	puella	1	F2-R2	KM276596	1	F1u-R1cm	KM276623	1	F4-R3	KM276640	1	F2-R4	KM276644	1	8F-382R	KM276652	1	1219F-1455R	KM276637
	pulchellum	1	F2-R2	KM276598	1	F1u-R1cm	KM276625	1	F4-R3	KM276641	1	F2-R4	KM276646	1	8F-382R	KM276650	1	1219F-1455R	KM276635
	scitulum				1	F1u-R1cm	KM276627	1	F4-R3	KM276643				1	8F-382R	KM276653			
	caerulescens	1	F2-R2	KM276597	1	F1u-R1cm	KM276624	1	F4-R3	KM276642	1	F2-R4	KM276645	1	8F-382R	KM276654	1	1219F-1455R	KM276636
Pseudagrion	*sublacteum*							1	F4-R3	KM276649									
Ischnura	*hastata*	9	F4-R1	KM276587-KM276595				16	F4-R3	KM276605-KM276620									
	cervula							1	F4-R3	KM276622									
	denticollis							1	F4-R3	KM276603									
	kellicotii							1	F4-R3	KM276621									
	posita							1	F4-R3	KM276604									
Protoneura	*capillaris*							1	F2-R3	KM276631									
	viridis							1	F2-R3	KM276632									
	sanguinipes							1	F2-R3	KM276634									
Microneura	*caligata*							1	F2-R3	KM276633									
Neoneura	*amelia*							2	F2-R3	KM276629-KM276630									
	maria							1	F2-R3	KM276628									
Hypolestes	*clara*							1	F3-R3	KM276601				1	8F-382R	KM276599			
	trinitatis				1	F2d-R1d	KM276648	1	F3-R3	KM276602				1	8F-382R	KM276600			
Calopteryx	*haemorrhoidalis*							3	F2-R3	KM276570-KM276572									
	splendens							5	F2-R3	KM276582-KM276586									
	virgo							6	F2-R3	KM276573-KM276578									
	xanthostoma							3	F2-R3	KM276579-KM276581									
Onychogomphus	*uncatus*							1	F2-R3	KM222659									
	forcipatus							1	F2-R3	KM222656									
	costae							1	F2-R3	KM222662									

Marker selection and development

As our goal was to identify putative single copy nuclear DNA sequence markers that were sufficiently polymorphic for phylogeographic studies we adopted an exon-primed, intron-crossing (EPIC) PCR strategy (Lessa, 1992). From an arthropod phylogenomic analysis of 62 single-copy nuclear protein-coding genes (Regier et al., 2010), which included the odonates *Ischnura verticalis* and *Libellula lydia*, we first selected the genes considered as "sufficiently variable (. . .) to be a candidate for testing across Lepidoptera" (Regier, 2007, pp. 15–33) and then identified those genes with introns in other insects using the BLAST (Altschul, Gish, Miller, Myers, & Lipman, 1990) tool in flybase (McQuilton, St Pierre, Thurmond, & FlyBase Consortium, 2012). We then downloaded sequence data from *I. verticalis* and *L. lydia* for this reduced set of nuclear genes from Genbank (http://www.ncbi.nlm.nih.gov/genbank). Due to apparently high polymorphism (Lee & Lin, 2012) we also downloaded the *Euphaea* sp. sequence data for myosin light chain (MLC), arrestin (ARR) and ferritin (FER) sequences from Genbank.

To obtain sequence data for primer design, we used a BLAST search (Altschul et al., 1990), performed in Geneious v.6.1.5 (http://www.geneious.com), to identify which of the candidate genes were present in a *C. mercuriale* transcriptome (derived from 454 sequencing of RNA expressed in larval thoracic muscle tissue; P.C. Watts and D.J. Thompson, unpublished). The putative locations of introns in the *C. mercuriale* transcriptome sequences were identified by comparison against homologous sequences from other insects. Primers around introns were designed manually in Bioedit v.5.0.9 (Hall, 1999).

Additionally, phosphoglucose isomerase gene (PGI) was examined as a candidate gene for PCR-optimization, and polymorphism evaluation in our target species using *Coenagrion* sp. specific primers (Fox, 2010), as it is a candidate locus for dispersal ability in insects (Wheat, Watt, Pollock, & Schulte, 2006). For comparison, we sequenced two widely used nuclear sequence markers: a fragment of 28S gene (Hasegawa & Kasuya, 2006) and a fragment of H3 (Ogden & Whiting, 2003).

Molecular biology and data analysis

Total genomic DNA was isolated from leg samples using a high-salt protocol (Sambrook & Russell, 2001). PCRs were carried out in 10 μl reaction volumes containing 5 μl of ReddyMix™ PCR Master Mix (Thermo Scientific, Waltham, Massachusetts, USA) [75 mM Tris-HCl (pH 8.8), 20 mM $(NH_4)_2SO_4$, 1.5 mM $MgCl_2$, 0.01% (v/v) Tween® 20, 0.2 mM each dNTP and 0.625 units ThermoPrime Taq DNA Polymerase], 1.5 mM $MgCl_2$, 0.2 pmol of each primer, and ~10 ng of genomic DNA. Thermal cycling conditions consisted of 5 min at 94°C, followed by 40 cycles of 30 s at 92°C, 30 s at T_a°C and 45 s at 72°C, and then 10 min at 72°C, where T_a is the marker-specific annealing temperature (Table 2).

Following PCR, the remaining primers were removed by incubation with 0.75 units Antarctic Phosphatase (New England Biolabs, Ipswich, Massachusetts, USA) and 0.6 units Exonuclease I (*E. coli*) (New England Biolabs) at 37°C for 75 min, followed by 15 min at 80°C [in 7 μl final reaction volumes containing 1X Exonuclease I Reaction Buffer (New England Biolabs)]. PCR products were sequenced in both directions using BigDye v.3.1 chemistry (Applied Biosystems, Foster City, California, USA), following the standard protocol but with 10 μl reaction volumes. Purified sequence products were run on an ABI3100xl (Applied Biosystems).

Chromatograms were checked for quality using Geneious v.6.1.5 (http://www.geneious.com) and nucleotide ambiguities with similar peak heights were considered to be heterozygous positions and were recoded with IUPAC ambiguity codes. Sequence alignment was conducted using MUSCLE (Edgar, 2004) (implemented in Geneious) with 10 as the maximum number of iterations, and alignments were then corrected manually if necessary. Haplotype reconstructions

Table 2. Details of PCR primers and thermal cycling conditions for successful amplification of regions of nuclear genes in odonates. T_a represents the marker-specific annealing temperature.

Gene (abbreviation)	Primer	Primer sequence 5'→3'	T_a (^{o}C)
Arginine methyltransferase (PRMT)	ARG_F2	TGC CGC CAA GGC TGG AGC ATC	50–53
	ARG_F3	CCG GAA CTC TAT GTA CCA CAA C	
	ARG_F4	TCG ACT CGT ATG CGC ATT TCG G	
	ARG_R3	TGC CAC CTT CCT AAT AGA GCT C	
Phosphoglucose isomerase (PGI)	Cp pgi 8 F	GTC GTG CAA AAC TCA CAG AAG AGG	56
	Cp pgi 382 R	TTC CTT CAT GTG ACT CAG AAC TGC	
	Cp pgi 1219 F	CTG CTG ACT TCA TAG CCC CTG TAA	56
	Cp pgi 1455 R	GGC CCC WAG AGT AAA AGG TGT GAC	
Myosin light chain (MLC)	Myo_F1u	ACT TCA CCC AAC TGC TCAC	47
	Myo_R1cm	CAT CAT CGA ATG ACT TGA	
	Myo_F2d	CAC CCA ACT GCT CAC MCT TT	50
	Myo_R1d	CAT CRT CGA ATG ACT TGA	
Cell division cycle 5 protein (CDC5)	CDC5_F4	GCT CGT TGG TAY GAA TGG TTA GAT CC	45
	CDC5_R1	GCT GGC TTT GTT TCT GGA TTA GGA TC	
	CDC5_F2	GCA AAA TTG ATG CCT ACM CAA TGG AG	45
	CDC5_R2	GGA TTA GGA TCA ATT TCT CCA GGC TTC	
Acetylglucosaminyl-transferase (AgT)	AgT_F2	TGC GTG CTC TTA ACC TTA GC	53
	AgT_R4	TAG GCT GAA GCT CAA TTG CTC	

were performed using PHASE v.2.1 (Stephens & Donnelly, 2003; Stephens, Smith, & Donnelly, 2001) implemented in DnaSP v.5 (Librado & Rozas, 2009). Unrooted haplotype networks were constructed using statistical parsimony (Templeton, Crandall, & Sing, 1992), to provide a visual comparison of the level of resolution of each genetic marker, using TCS v.1.21 (Clement, Posada, & Crandall, 2000), with indels treated as a fifth character state.

We calculated standard summary statistics of diversity (S, number of segregating sites; π, nucleotide diversity; h, number of haplotypes; Hd, haplotype diversity), the minimum number of recombination events (R_M) (Hudson & Kaplan, 1985) and respective statistical confidence based on a coalescent simulation algorithm, and tested for deviation from neutral predictions using Tajima's test (D) (Tajima, 1989) using the phased data in DNAsp v.5.10.01 (Librado & Rozas, 2009). The phi-test (Bruen, Philippe, & Bryant, 2006) was performed to assess statistical evidence for recombination in SplitsTree v.4.13.1 (Huson & Bryant, 2006).

Results

Sixteen of the putative variable genes from Regier (2007) had one or more confirmed introns in insects. We found six of these genes [EF-1α, cell division cycle 5 protein (CDC5), arginine methyltransferase (PRMT), acetylglucosaminyl-transferase (AgT), alanyl-tRNA synthetase, and transmembrane protein] in the *C. mercuriale* transcriptome, as well as sequences of the myosin light chain (MLC) and phosphoglucose isomerase (PGI). Sixty-five primers were designed for regions within these eight genes. We achieved successful PCR-amplification from *C. mercuriale* genomic DNA in five genes: CDC5, PRMT, AgT, MLC and PGI (Tables 1, 2,3). We sequenced the longest fragments that could be amplified for each gene, although for the PGI locus two non-overlapping fragments (PGI-8, PGI-1219) were sequenced (see Tables 1, 2, Figure 1).

The five new nuclear loci sequenced present distinct structural features regarding fragment length, presence of introns and presence of indels (Table 3, Figure 1). The new markers are capable of amplifying some 3200 bp in *C. mercuriale*, representing fragments ranging from ~200 bp (CDC5) to ~750 bp (PRMT and PGI-1219) (Table 3, Figure 1). In *C. mercuriale*, the fragment length varies in four loci due to the presence of indels, with the longest indel being 6 bp long in

Table 3. Summary diversity statistics for eight nuclear sequence markers in 10 specimens of *Coenagrion mercuriale* representing individuals from Europe ($n = 5$) and North Africa ($n = 5$).

Gene	FW primer pair	RV primer	Length (bp)	Indel no./ length (bp)	Intron no./ length (bp)	S	π	h	Hd	R_M	ϕ_w	D
28S[A]	28sf	28sr	830	0	0	0	0	1-1	0	NA	NA	NA
H3[B]	HexAF	HexAR	284	0	0	2	0.0038	2-2	0.556	0	1.000 ns	1.8443 ns
CDC5	CDC5_F2	CDC5_R2	208	0	1 (88)	5	0.0049	6	0.795	0	1.000 ns	−0.3060 ns
MLC	Myo_F1u	Myo_R1cm	319	1 (1)	1 (257)	16	0.0170	8-8	0.821	0	1.000 ns	0.8076 ns
PRMT	ARG_F4	ARG_R3	759	1 (1)	3 (97, 78, 89)	17	0.0090	8-9	0.874	0	1.000 ns	1.3426 ns
AgT	AgT_F2	AgT_R4	603	3 (1, 1, 4)	1 (518)	22	0.0134	8-10	0.884	2*	0.229 ns	1.2025 ns
PGI	Cp pgi 1219F	Cp pgi 1455R	746	3 (6, 1, 2)	1 (>612)	17	0.0091	7-7	0.832	0	1.000 ns	1.4732 ns
PGI	Cp pgi 8F	Cp pgi 382R	619	0	2 (145, 171)	8	0.0055	6-6	0.758	0	1.000 ns	1.3260 ns

Length, length of sequence in the final alignment (bp); Indel no., the number of insertion/deletions polymorphisms and respective length; Intron no. the number of introns and respective length(s); S, number of segregating sites; π, nucleotide diversity; h, number of haplotypes (calculated by DNAsp and TCS respectively); Hd, haplotype diversity; R_M, minimum number of recombination events; ϕ_w, phi-test; D, Tajima's test. ns, not significant; $^*p < 0.05$.

[A] Hasegawa & Kasuya (2006)

[B] Ogden & Whiting (2003)

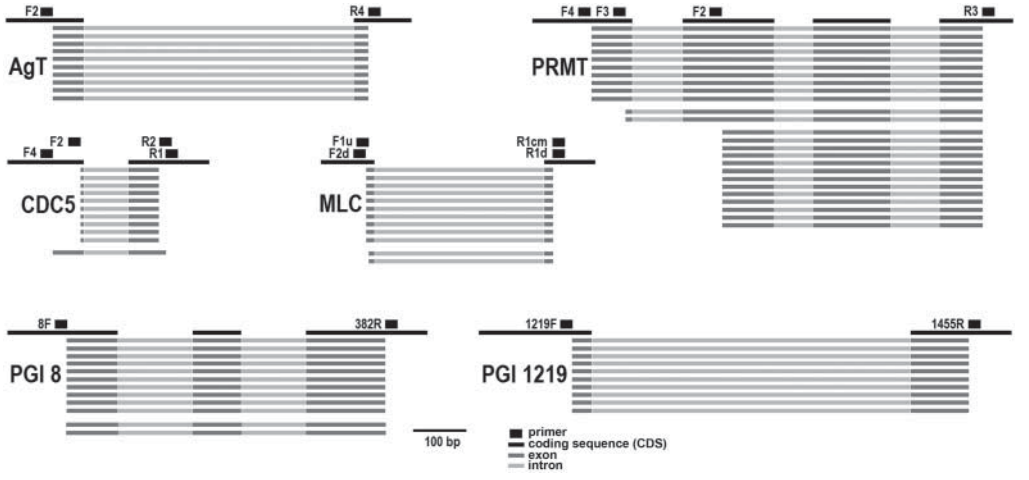


Figure 1. Diagrammatic representation of the six fragments of the five new, nuclear loci to indicate the locations of primer annealing sites relative to the exons and introns. The coding sequence (solid black line) represents *C. mercuriale* transcriptome sequence, while exons (dark grey) and introns (light grey) represent new sequence data. The first 10 lines represent individuals of *C. mercuriale*, whereas each of the following lines represents successful amplification of other species sequences during cross-amplification tests (see Table 1 for details on species).

PGI-1219 (Table 3). As we followed an EPIC strategy, all new markers encompass between one and three introns, which represent the location of most polymorphisms, with the smallest intron being 78 bp in length (in PRMT) and the longest intron more than 612 bp (in PGI-1219) (Table 3, Figure 1).

All new markers exhibited instances of heterozygous individuals (in the *C. mercuriale* dataset) and presented a significant increase in polymorphism when compared with the commonly used nuclear fragments (28S and H3) (Table 3, Figure 2); thus the new loci provide good phylogeographic resolution. Among the 10 European and North African *C. mercuriale*, the new markers yielded between two and five haplotypes within regions, which contrasts with the 28S and H3 fragments where there were just one or two haplotypes in total (Figure 2). Moreover, the European and North African samples are genetically distinct, and reasonably divergent, at all new markers, except CDC5 in which European and North African specimens share the most common (possibly ancestral) haplotype (Figure 2).

No deviation from neutral predictions was apparent at any locus (i.e. Tajima's *D*), although the sample size is small (Table 3). Recombination was detected at AgT, with a significant ($p < 0.05$ after coalescence simulations) minimum number of recombination events of 2, although the phi-test did not uncover evidence of recombination in any locus (Table 3).

Successful cross-amplification and sequence data were obtained for all new markers for four species of *Coenagrion*, using the same primers that proved optimal for *C. mercuriale* (Table 1) with *C. scitulum* failing to amplify two fragments (AgT and PGI-1219). Arginine methyltransferase (PRMT) was successfully amplified in all 27 odonates species examined. The PCR primer pairs that amplify this locus in coenagrionids and *Hypolestes* species generates fragments of ∼760 bp and ∼700 bp in length, respectively, and which encompasses three introns (Table 3, Figure 1). In all other species, including the anisopterans, successful PCR amplification of PRMT could only be achieved with the primer pair that targets a smaller (∼500 bp) fragment that encompasses two introns (Table 3). Other loci also amplified fragments in non-coenagrionids, most notably MLC and PGI-8 in both *Hypolestes* species, reinforcing the potential for the use of these loci in other odonates (Table 1).

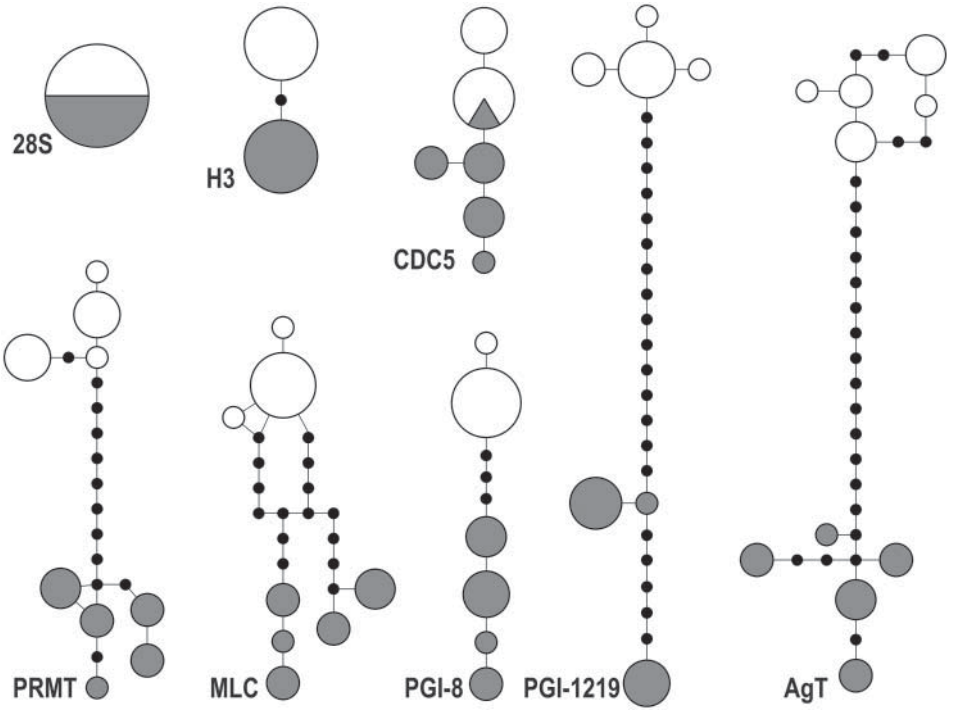


Figure 2. Haplotype networks (generated using TCS v.1.21) indicating diversity and relationships among *Coenagrion mercuriale* from Europe (white, $n = 5$) and North Africa (grey, $n = 5$) to illustrate the level of phylogeographic resolution yielded by eight nuclear DNA markers (see Tables 1, 2 and 3 for details of loci). Circle size is proportional to haplotype frequencies and black points represent missing or unsampled haplotypes.

Discussion

Phylogeographic studies of odonates remain relatively scarce albeit with increasing prominence in recent years (e.g. Artiss, 2004; Bernard et al., 2011; Büsse et al., 2012; Hayashi, Dobata, & Futahashi, 2005; Jordan, Simon, Foote, & Englund, 2005; Kiyoshi, 2008; Kiyoshi & Sota, 2006). As phylogeographic analyses of odonates typically rely on mtDNA markers, they leave unexplored the type of information and associated advantages that can be derived from nuclear markers. But the crucial need for independent markers is highlighted by reports that phylogeographic patterns based on mtDNA markers can be incongruent with those derived from nuclear markers (e.g. Hayashi et al., 2005). The absence of a set of independent DNA markers with appropriate levels of polymorphism has been an apparent constraint for making robust studies of intraspecific diversity in odonates. Using an exon-primed, intron-crossing (EPIC) PCR strategy we developed five new, polymorphic nuclear DNA sequence loci to improve the resolution of phylogeographic studies of coenagrionids and other odonates.

Besides the key issue of marker independence, the use of nuclear DNA sequence markers allows the detection of hybrids and of introgressive hybridization events between two or more taxa (Hayashi et al., 2005), and of gene flow between populations (Alves et al., 2006; Ballard & Whitlock, 2004; Sánchez-Guillén, Wellenreuther, Cordero-Rivera, & Hansson, 2011). Moreover, phylogeographic studies of odonates have increased potential interest in the use of nuclear DNA sequence markers, as nuclear markers display a higher average diversity in invertebrates than in vertebrates (Bazin, Glémin, & Galtier, 2006).

Although the Coenagrionidae is the most diverse zygopteran family, surprisingly little is known about phylogeographic patterns of coenagrionids (but see Bernard et al., 2011; Jordan et al., 2005; Swaegers et al., 2014). Also, the group phylogeny remains a source of debate (Carle et al., 2008; Dijkstra et al., 2014; Dumont, Vierstraete, & Vanfleteren, 2010), highlighting the need for the development of new markers. Using these new nuclear markers in *C. mercuriale*, we uncovered high levels of polymorphism and considerable divergence among two geographic groups, a diversity that was not detected using 28S and H3. Polymorphism levels were higher in markers with greater length of intron in the fragment, underlining the perceived usefulness of sequencing introns for phylogeographic studies. On the other hand, as the primers are designed in exon regions (more conserved than introns) they can be potentially applied across a wide taxonomic range.

The cross-amplification capacity of primers specifically designed for a species, in this case *C. mercuriale*, is expected to decrease with increasing genetic distance due to mutations in the primer sites, and is connected with the level of conservation of the exon fragment. This may explain why we could not obtain fragments of AgT and PGI-1219 for *C. scitulum*, while primers for PRMT amplified fragments from the genomes of zygopterans from three other families and even worked in species from a highly divergent group of gomphids (Table 3). Another possible explanation is that the targeted fragments of AgT and PGI-1219 in *C. scitulum* may be too long due to the presence of insertion(s) causing failure of amplification. Nevertheless, and given that more organisms have their genomes sequenced, the design of primers for these markers in other Odonata species would circumvent many of these potential limitations, providing a powerful and accessible tool for future studies.

Moreover, Odonata phylogenetics and systematics studies would also benefit from an increase in the number of nuclear markers, as presently the markers available have recognized limited resolution power and many phylogenetic relations await clarification (Ballare & Ware, 2011; Blanke, Greve, Mokso, Beckman, & Misof, 2013; Dijkstra et al., 2014; Kim et al., 2014).

In summary, we have developed five new nuclear polymorphic loci that can be used to improve our understanding of phylogeographic patterns of many coenagrionids in future studies, and one new locus that works on a wide taxonomic range of odonates. Odonata phylogeographic studies need to use several independent polymorphic nuclear DNA markers to provide sufficient resolution and statistical robustness to make novel insights into the evolutionary history and conservation genetics studies of dragonflies and damselflies.

Acknowledgements

The authors are grateful to their colleagues who sent samples for the genetic analysis: Jean-Pierre Boudot, Sonke Hardersen, Elisa Riservato and Janne Swaegers. SF would particularly like to acknowledge Bruno Carvalho, Arie van der Meijden and Diana Pedroso for productive brainstorming discussions during the early stages of this work. Sample collection benefited from fieldwork funded by the project "*Coenagrion mercuriale*: distribución, diversidad genética y estado de conservación" of Instituto de Estudios Ceutíes (Ceuta – Spain). SF is supported by Fundação para a Ciência e Tecnologia (SFRH/BD/65038/2009). YTC was supported by a project "Ciencia y Conciencia" funded by Universidad de Oriente, Cuba (project code 9617) and a WDA Conservation and Research Grant. ACR and MOLC were supported by grants from the Spanish Ministry with competence in Science, which included FEDER funds (CGL2010-11959-E and CGL2011-22629).

Disclosure statement

The authors declare that no financial interest or benefit exists arising from the direct applications of their research.

References

Altschul, S. F., Gish, W., Miller, W., Myers, E. W., & Lipman, D. J. (1990). Basic local alignment search tool. *Journal of Molecular Biology, 215*, 403–410. doi:10.1016/s0022-2836(05)80360-2

Alves, P. C., Harris, D. J., Melo-Ferreira, J., Branco, M., Suchentrunk, F., Boursot, P., & Ferrand, N. (2006). Hares on thin ice: introgression of mitochondrial DNA in hares and its implications for recent phylogenetic analyses. *Molecular Phylogenetics and Evolution, 40*, 640–641. doi:10.1016/j.ympev.2006.02.016

Artiss, T. (2004). Phylogeography of a facultatively migratory dragonfly, *Libellula quadrimaculata* (Odonata: Anisoptera). *Hydrobiologia, 515*, 225–234. doi:10.1023/b:hydr.0000027332.57786.9d

Aurelle, D., Ledoux, J.-B., Rocher, C., Boesa, P., Chenuil, A., Féral, J.-P. (2011). Phylogeography of the red coral (*Corallium rubrum*): inferences on the evolutionary history of a temperate gorgonian. *Genetica, 139*, 855–869. doi:10.1007/s10709-011-9589-6

Avise, J. C. (2000). *Phylogeography: the history and formation of species*. Cambridge, MA: Harvard University Press.

Avise, J. C. (2009) Phylogeography: retrospect and prospect. *Journal of Biogeography, 36*, 3–15. doi:10.1111/j.1365-2699.2008.02032.x

Ballard, J. W. O., & Whitlock, M. C. (2004). The incomplete natural history of mitochondria. *Molecular Ecology, 13*, 729–744. doi:10.1046/j.1365-294x.2003.02063.x

Ballare, E., & Ware, J. L. (2011). Dragons fly, biologists classify: an overview of molecular odonate studies, and our evolutionary understanding of dragonfly and damselfly (Insecta: Odonata) behavior. *International Journal of Odonatology, 14*, 137–147. doi:10.1080/13887890.2011.579538

Bazin, E., Glémin, S., & Galtier, N. (2006). Population size does not influence mitochondrial genetic diversity in animals. *Science, 312*, 570–571. doi:10.1126/science.1122033

Bernard, R., Heiser, M., Hochkirch, A., & Schmitt, T. (2011). Genetic homogeneity of the Sedgling *Nehalennia speciosa* (Odonata: Coenagrionidae) indicates a single Würm glacial refugium and trans-Palaearctic postglacial expansion. *Journal of Zoological Systematics and Evolutionary Research, 49*, 292–297. doi:10.1111/j.1439-0469.2011.00630.x

Blanke, A., Greve, C., Mokso, R., Beckman, F., & Misof, B. (2013). An updated phylogeny of Anisoptera including formal convergence analysis of morphological characters. *Systematic Entomology, 38*, 474–490. doi:10.1111/syen.12012

Bruen, T. C., Philippe, H., Bryant, D. (2006). A simple and robust statistical test for detecting the presence of recombination. *Genetics, 172*, 2665–2681. doi:10.1534/genetics.105.048975

Büsse, S., von Grumbkow, P., Hummel, S., Shah, D. N., Tachamo Shah, R. D., Li, J., Zhang, X., Yoshizawa, K., Wedmann, S., Hörnschemeyer, T. (2012). Phylogeographic analysis elucidates the influence of the ice ages on the disjunct distribution of relict dragonflies in Asia. *PLoS ONE, 7*, e38132. doi:10.1371/journal.pone.0038132

Carle, F. L., Kjer, K. M., & May, M. L. (2008). Evolution of Odonata, with special reference to Coenagrionoidea (Zygoptera). *Arthropod Systematics and Phylogeny, 66*, 37–44.

Chen, M. Y., Chaw, S. M., Wang, J. F., Villanueva, R. J., Nuñeza, O. M., & Lin, C. P. (2014). Mitochondrial genome of a flashwing demoiselle, *Vestalis melania* from the Philippine Archipelago. *Mitochondrial DNA*. doi:10.3109/19401736.2013.845757

Clement, M., Posada, D., & Crandall, K. A. (2000). TCS: a computer program to estimate gene genealogies. *Molecular Ecology, 9*, 1657–1660. doi:10.1046/j.1365-294x.2000.01020.x

Cruickshank, R. H. (2002). Molecular markers for the phylogenetics of mites and ticks. *Systematic and Applied Acarology, 7*, 3–14.

Dijkstra, K.-D. B., & Kalkman, V. J. (2012). Phylogeny, classification and taxonomy of European dragonflies and damselflies (Odonata): a review. *Organisms Diversity & Evolution, 12*, 209–227. doi:10.1007/s13127-012-0080-8

Dijkstra, K.-D. B., Kalkman, V. J., Dow, R. A., Stokvis, F. R., & van Tol, J. (2014). Redefining the damselfly families: the first comprehensive molecular phylogeny of Zygoptera (Odonata). *Systematic Entomology, 39*, 68–96. doi:10.1111/syen.12035

Dijkstra, K.-D. B., & Lewington, R. (2006). *Field guide to the Dragonflies of Britain and Europe.* Gillingham, Dorset: British Wildlife Publishing.

Dumont, H. J., Vanfleteren, J. R., De Jonckheere, J. F., & Weekers, P. H. H. (2005). Phylogenetic relationships, divergence time estimation, and global biogeographic patterns of Calopterygoid damselflies (Odonata: Zygoptera) inferred from ribosomal DNA sequences. *Systematic Biology, 54*, 347–362. doi:10.1080/10635150590949869

Dumont, H. J., Vierstraete, A., & Vanfleteren, J. R. (2010). A molecular phylogeny of the Odonata (Insecta).*Systematic Entomology, 35*, 6–18. doi:10.1111/j.1365-3113.2009.00489.x

Edgar, R. C. (2004). MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Research, 32*, 1792–1797. doi:10.1093/nar/gkh340

Fox, G. (2010). *The genetic basis of dispersal traits in the damselfly* Coenagrion puella (Unpublished MSc Research Project). Liverpool: University of Liverpool.

Galtier, N., Nabholz, B., Glémin, S., & Hurst, G. D. D. (2009). Mitochondrial DNA as a marker of molecular diversity: a reappraisal. *Molecular Ecology, 18*, 4541–4550. doi:10.1111/j.1365-294x.2009.04380.x

Hall, T. A. (1999). Bioedit: a user friendly biological sequence alignment editor and analysis program for Windows 95/98/NT. *Nucleic Acids Symposium Series, 41*, 95–98.

Hare, M. P. (2001). Prospects for nuclear gene phylogeography. *Trends in Ecology and Evolution, 16*, 700–706. doi:10.1016/s0169-5347(01)02326-6

Harris, D. J., & Crandall, K. A. (2000). Intragenomic variation within ITS1 and ITS2 of freshwater crayfishes (Decapoda: Cambaridae): Implications for phylogenetic and microsatellite studies. *Molecular Biology and Evolution, 17*, 284–291. doi:10.1093/oxfordjournals.molbev.a026308

Hasegawa, E., & Kasuya, E. (2006). Phylogenetic analysis of the insect order Odonata using 28S and 16S rDNA sequences: a comparison between data sets with different evolutionary rates. *Entomological Science, 9*, 55–66. doi:10.1111/j.1479-8298.2006.00154.x

Hayashi, F., Dobata, S., & Futahashi, R. (2005). Disturbed population genetics: suspected introgressive hybridization between two *Mnais* damselfly species (Odonata). *Zoological Science, 22*, 869-881. doi:10.2108/zsj.22.869

Hudson, R. R., & Kaplan, N. L. (1985). Statistical properties of the number of recombination events in the history of a sample of DNA sequences. *Genetics, 111*, 147–164. Retrieved from http://www.genetics.org/content/111/1/147.abstract

Hurst, G. D. D., & Jiggins, F. M. (2005). Problems with mitochondrial DNA as a marker in population, phylogeographic and phylogenetic studies: the effects of inherited symbionts. *Proceedings of the Royal Society B: Biological Sciences, 272*, 1525–1534. doi:10.1098/rspb.2005.3056

Huson, D. H., & Bryant, D. (2006). Application of phylogenetic networks in evolutionary studies. *Molecular Biology and Evolution, 23*, 254–267. doi:10.1093/molbev/msj030

Jennings, R. M., Etter, R. J., & Ficarra, L. (2013). Population differentiation and species formation in the deep sea: the potential role of environmental gradients and depth. *PLoS ONE, 8*, e77594. doi:10.1371/journal.pone.0077594

Jiang, H., Barker, S. C., & Shao, R. (2013). Substantial variation in the extent of mitochondrial genome fragmentation among blood-sucking lice of mammals. *Genome Biology and Evolution, 5*, 1298–1308. doi:10.1093/gbe/evt094

Jordan, S., Simon, C., Foote, D., & Englund, R. A. (2005) Phylogeographic patterns of Hawaiian *Megalagrion*damselflies (Odonata: Coenagrionidae) correlate with Pleistocene island boundaries. *Molecular Ecology, 14*, 3457–3470. doi:10.1111/j.1365-294x.2005.02669.x

Jordan, S., Simon, C., & Polhemus, D. (2003). Molecular systematics and adaptive radiation of Hawaii's endemic Damselfly genus *Megalagrion* (Odonata: Coenagrionidae). *Systematic Biology, 52*, 89–109.

Kalkman, V. J., Boudot, J.-P., Bernard, R., Conze, K.-J., De Knijf, G., Dyatlova, E., Ferreira, S., Joviæ, M., Ott, J., Riservato, E., & Sahlén, G. (2010). European Red List of dragonflies. Luxembourg: Publications Office of the European Union.

Kim, M. J., Jung, K. S., Park, N. S., Wan, X., Kim K.-G., Jun, J., Yoon, T. J., Bae, Y. J., Lee, S. M., & Kim, I. (2014). Molecular phylogeny of the higher taxa of Odonata (Insecta) inferred from COI, 16S rRNA, 28S rRNA, and EF1-α sequences. *Entomological Research, 44*, 65–79. doi:10.1111/1748-5967.12051

Kiyoshi, T. (2008). Differentiation of golden-ringed dragonfly *Anotogaster sieboldii* (Selys, 1854) (Cordulegastridae: Odonata) in the insular East Asia revealed by the mitochondrial gene genealogy with taxonomic implications. *Journal of Zoological Systematics and Evolutionary Research, 46*, 105–109. doi:10.1111/j.1439-0469.2007.00445.x

Kiyoshi, T., & Sota, T. (2006). Differentiation of the dragonfly genus*Davidius* (Odonata: Gomphidae) in Japan inferred from mitochondrial and nuclear gene genealogies. *Zoological Science, 23*, 1–8. doi:10.2108/zsj.23.1

Lee, E. M., Hong, M. Y., Kim, M. I., Kim, M. J., Park, H. C., Kim, K. Y., Lee, I. H., Bae, C. H., Jin, B. R., Kim, I. (2009). The complete mitogenome sequences of the palaeopteran insects *Ephemera orientalis* (Ephemeroptera: Ephemeridae) and *Davidius lunatus* (Odonata: Gomphidae). *Genome, 52*, 810–817. doi:10.1139/g09-055

Lee, Y.-H., & Lin, C.-P. (2012). Pleistocene speciation with and without gene flow in *Euphaea*damselflies of subtropical and tropical East Asian islands. *Molecular Ecology, 21*, 3739–3756. doi:10.1111/j.1365-294x.2012.05654.x

Lessa, E. P. (1992). Rapid surveying of DNA sequence variation in natural populations. *Molecular Biology and Evolution, 9*, 323–330. Retrieved from http://mbe.oxfordjournals.org/content/9/2/323.abstract

Librado, P., & Rozas, J. (2009). DnaSP v5: a software for comprehensive analysis of DNA polymorphism data. *Bioinformatics*, 25, 1451–1452. doi:10.1093/bioinformatics/btp187

Lin, C.-P., Chen, M. Y., & Huang, J. P. (2010). The complete mitochondrial genome and phylogenomics of a damselfly, *Euphaea formosa* support a basal Odonata within the Pterygota. *Gene, 468*, 20–29. doi:10.1016/j.gene.2010.08.001

Lohse, K., Sharanowski, B., & Stone, G. N. (2010). Quantifying the Pleistocene history of the oak gall parasitoid Cecidostiba fungosa. *Evolution, 64*, 2664–2681. doi:10.1111/j.1558-5646.2010.01024.x

Lopez, J.V., Yuhki, N., Masuda, R., Modi, W., & O'Brien, S.J. (1994). Numt, a recent transfer and tandem amplification of mitochondrial DNA to the nuclear genome of the domestic cat. *Journal of Molecular Evolution, 39*, 174–190. Retrieved from http://link.springer.com/article/10.1007%2FBF00163806

Lorenzo-Carballa, M. O., Thompson, D. J., Cordero-Rivera, A., & Watts, P. C. (2013). Next generation sequencing yields the complete mitochondrial genome of the scarce blue-tailed damselfly, *Ischnura pumilio*. *Mitochondrial DNA, 25*, 247–248. doi:10.3109/19401736.2013.796518.

McQuilton, P., St Pierre, S. E., Thurmond, J., & FlyBase Consortium (2012). FlyBase 101 – the basics of navigating FlyBase. *Nucleic Acids Research, 40*, D706–D714. doi:10.1093/nar/gkr1030

Miraldo, A., Hewitt, G. M., Dear, P. H., Paulo, O. S., & Emerson, B. C. (2012). Numts help to reconstruct the demographic history of the ocellated lizard (*Lacerta lepida*) in a secondary contact zone. *Molecular Ecology*, 21, 1005–1018. doi:10.1111/j.1365-294X.2011.05422.x

Molecular Ecology Resources Primer Development Consortium, Bonizzoni, M., Bourjea, J., Chen, B., Crain, B. J., Cui, L., Fiorentino, V., Hartmann, S., Hendricks, S., Ketmaier, V., Ma, X., Muths, D., Pavesi, L., Pfautsch, S., Rieger, M. A., Santonastaso, T., Sattabongkot, J., Taron, C. H., Taron, D. J., Tiedemann, R., Yan, G., Zheng, B., & Zhong, D. (2012). Permanent genetic resources added to molecular ecology resources database. 1 August 2011–30 September 2011. *Molecular Ecology Resources, 12*, 185–189. doi:10.1111/j.1755-0998.2011.03046.x

Moritz, C. (2002). Strategies to protect biological diversity and the evolutionary processes that sustain it. *Systematic Biology, 51*, 238–254. Retrieved from http://sysbio.oxfordjournals.org/content/51/2/238.long

Ogden, T. H., & Whiting, M. F. (2003). The problem with "the Paleoptera Problem": sense and sensitivity. *Cladistics, 19*, 432–442. doi:10.1111/j.1096-0031.2003.tb00313.x

Regier, J. C. (2007). *Protocols, concepts, and reagents for preparing DNA sequencing templates.*Version 9/19/07. Retrieved from http://www.umbi.umd.edu/users/jcrlab/PCR_primers.pdf

Regier, J. C., Shultz, J. W., Zwick, A., Hussey, A., Ball, B., Wetzer, R., Martin, J. W., & Cunningham, C. W. (2010). Arthropod relationships revealed by phylogenomic analysis of nuclear protein-coding sequences. *Nature, 463*, 1079–1083. doi:10.1038/nature08742

Sambrook, J., & Russell, D. W. (2001). *Molecular cloning: a laboratory manual*. 3rd edition. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press.

Samraoui, B., Boudot, J-P., Ferreira, S., Riservato, E., Jovic, M., Kalkman, V. J., & Schneider, W. (2010). The Odonata of North Africa: Red List status and distribution. In: García N, Cuttelod A, Abdul Malak D, editors. *The status and distribution of freshwater biodiversity in Northern Africa* (p. 141). Gland: IUCN.

Sánchez-Guillén, R. A., Wellenreuther, M., Cordero-Rivera, A., & Hansson, B. (2011). Introgression and rapid species turnover in sympatric damselflies. *BMC Evolutionary Biology, 11*, 210. doi:10.1186/1471-2148-11-210

Simon, C., Frati, F., Beckenbach, A., Crespi, B., Liu, H., & Flook P. (1994). Evolution, weighting, and phylogenetic utility of mitochondrial gene-sequences and a compilation of conserved polymerase chain-reaction primers. *Annals of the Entomological Society of America, 87*, 651–701.

Simon, S., Schierwater, B., & Hadrys, H. (2010). On the value of elongation factor-1α for reconstructing pterygote insect phylogeny. *Molecular Phylogenetics and Evolution, 54*, 651–656. doi:10.1016/j.ympev.2009.09.029

Slade, R. W., Moritz, C., Heideman, A., & Hale, P. T. (1993). Rapid assessment of single-copy nuclear DNA variation in diverse species. *Molecular Ecology, 2*, 359–373. doi:10.1111/j.1365-294x.1993.tb00029.x

Stephens, M., & Donnelly, P. (2003). A comparison of Bayesian methods for haplotype reconstruction from population genotype data. *American Journal of Human Genetics, 73*, 1162–1169. doi:10.1086/379378

Stephens, M., Smith, N., & Donnelly, P. (2001). A new statistical method for haplotype reconstruction from population data. *American Journal of Human Genetics, 68*, 978–989. doi:10.1086/319501

Swaegers, J., Mergeay, J., Therry, L., Bonte, D., Larmuseau, M. H. D., & Stoks, R. (2014). Unravelling the effects of contemporary and historical range expansion on the distribution of genetic diversity in the damselfly *Coenagrion scitulum*. *Journal of Evolutionary Biology, 27*, 748–759. doi:10.1111/jeb.12347

Tajima, F. (1989). Statistical method for testing the neutral mutation hypothesis by DNA polymorphism. *Genetics, 123*, 585–595.

Templeton, A. R., Crandall, K. A., & Sing, C. F. (1992). A cladistic analysis of phenotypic associations with haplotypes inferred from restriction endonuclease mapping and DNA sequence data. III. Cladogram estimation. *Genetics, 132*, 619–633.

Wang, J.-F., Chen, M.-Y., Chaw, S.-M., Morii, Y., Yoshimura, M., Sota, T., & Lin C.-P. (2014). Complete mitochondrial genome of an enigmatic dragonfly, *Epiophlebia superstes* (Odonata, Epiophlebiidae). *Mitochondrial DNA*, doi:10.3109/19401736.2013.845756

Weekers, P. H. H., De Jonckheere, J. F., & Dumont, H. J. (2001). Phylogenetic relationships inferred from ribosomal ITS sequences and biogeographic patterns in representatives of the genus *Calopteryx* (Insecta: Odonata) of the West Mediterranean and adjacent West European zone. *Molecular Phylogenetics and Evolution, 20*, 89–99. doi:10.1006/mpev.2001.0947

Wheat, C. W., Watt, W. B., Pollock, D. D., & Schulte, P. M. (2006). From DNA to fitness differences: Sequences and structures of adaptive variants of *Colias*phosphoglucose isomerase (PGI). *Molecular Biology and Evolution, 23*, 499–512. doi:10.1093/molbev/msj062